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CARBOHYDRATE METABOLISM OF THE DEVELOPING EGG AND EMBRYO[1]

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Studies on the respiration of intact eggs and isolated embryos of the grasshopper, *Melanoplus differentialis,* reveal that the functional intensity of the enzymes concerned with aerobic metabolism is altered considerably during different stages of embryonic development (Bodine and Boell, 1934; Bodine and Boell, 1936; Boell, 1935; Bodine and Lu, 1950a, 1950b; Bodine, Lu and West, 1952). The question of the nature and relative amounts of various metabolites used during embryonic development has attracted the attention of many investigators using a diversity of biological materials (Spratt, 1952). Needham (1942) has summarized large amounts of data and introduced a concept of a definite sequence in which metabolites are used during the course of embryonic development. It is also generally accepted that the terrestrial cleidoic egg consumes large amounts of fat and a much smaller amount of protein and carbohydrate during development. Results previously recorded in this laboratory seem to support these above views (Boell, 1935; Hill, 1945). Much of the evidence pertaining to metabolism during embryonic development has been obtained from data on the intact egg and little from studies of the intact embryo freed of yolk.

The importance of carbohydrates as energy sources in biological systems is well known. The present investigation is the first of a series planned to determine the importance of carbohydrate in the mechanism of the gross chemical transformations from yolk to embryo in the developing egg of the grasshopper.

Methods

Embryos of the grasshopper, *Melanoplus differentialis,* were obtained, yolk free, by previously described methods of Bodine and Boell (1934, 1936).

Fractionation of the embryos (Bodine and Lu, 1950a, 1951) was carried out either in Ringer solution buffered with 1/15 *M* phosphates (pH 6.8) or in 0.25 *M* sucrose containing 0.0035 *M* magnesium and calcium chlorides. No quantitative differences in results were observed between the two media and the latter was the one of choice since the morphological integrity of the intracellular particles seemed

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better preserved in it. The calcium ion in the sucrose medium was necessary to prevent clumping of nuclei and destruction of the nuclear membranes. The magnesium ion was found essential for maximum reactions of homogenates.

Respiration experiments were carried out using standard Warburg manometers. Seven experimental manometers contained 2,2,4-trimethyl pentane colored with Sudan IV as a manometer fluid and seven similar ones contained Brodie's solution. Both sets of manometers were carefully checked against each other. The sensitivity of the manometers was greatly increased by the use of the 2,2,4-trimethyl pentane as manometer fluid.

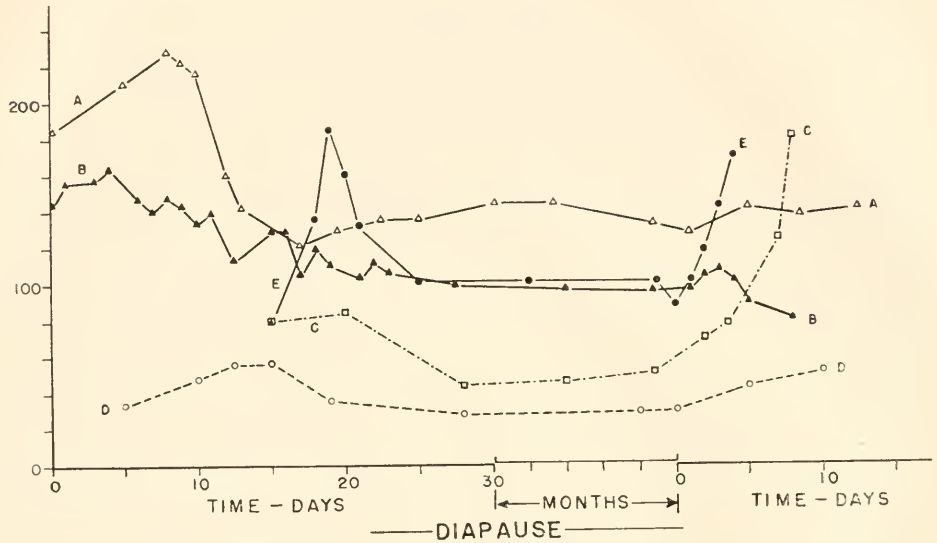

FIGURE 1. Shows total amount of carbohydrate per intact whole egg (A), and per yolk of whole egg (B); hexose content of embryo (C), and yolk (D), throughout course of embryonic development. Curve (E) shows endogenous O_2 uptake of embryo homogenates, taking that for the diapause embryo homogenate as 100%. Abscissa, time in days and months; ordinate micrograms per egg or embryo for carbohydrates, for O_2 uptake, percentage. Values for hexoses of embryo are increased by 1/10 over actual values found. Each point represents averages from many experiments.

Commercial glucose, glucose-1-phosphate (dipotassium salt), fructose-6-phosphate (dibarium salt converted to the sodium or potassium salt) and fructose 1,6-diphosphate (dibarium salt converted to sodium salt) obtained from the Swartz laboratories, were employed as substrates. The substrates (0.5 cc.) were tipped from the side arms of the manometer flasks at the end of a 40-minute control period. The final concentration of the substrates per 1.5 ml. (total volume of fluid in flask) was approximately 40 moles $\cong$ 0.026 M for glucose, while fructose 1,6-diphosphate contained the same carbohydrate content per cc. as did glucose-1-phosphate.

Carbohydrate determinations were made with the commercial product "Microne" obtained from the National Biochemical Laboratory. "Microne" is chemically anthrone and was described by Dreywood (1946) as a specific test for carbohydrate. The maximum absorption of the green color developed using a glucose test solution

and 0.2% "Microne" in 95% sulfuric acid was found to be 625 mμ which is similar to that reported by Viles and Silverman (1949). "Microne" is used as a microcolorimetric method for determination of sugars, starches and celluloses. This reagent is of interest since equivalent amounts of glucose, glycogen and fructose

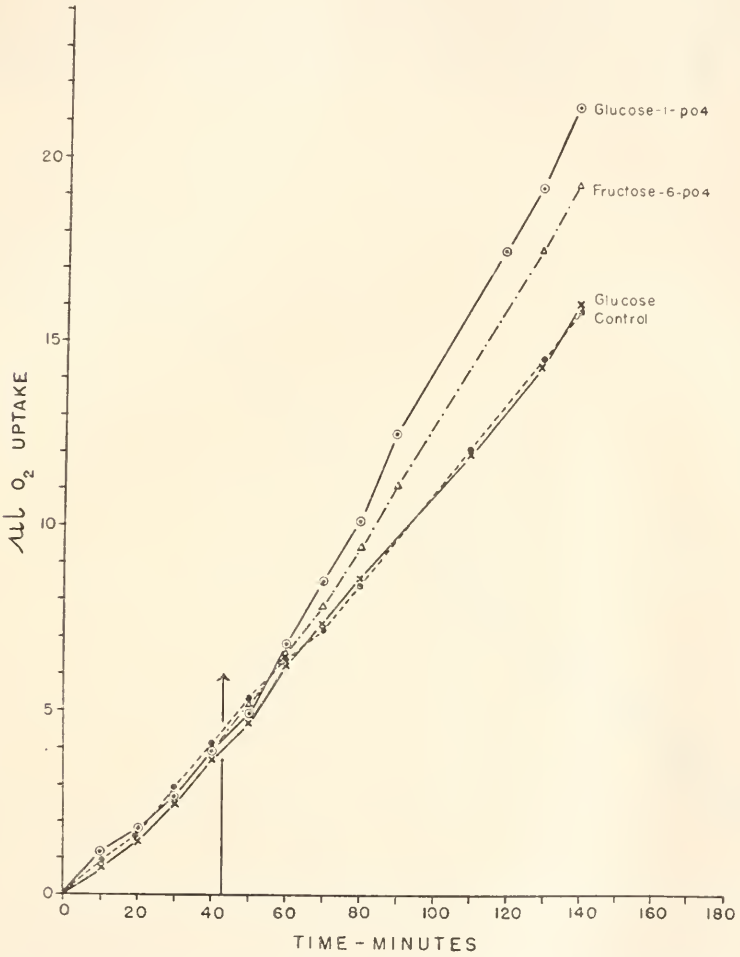

FIGURE 2. Shows endogenous O_2 uptake of diapause embryo suspended in different substrates. Arrow indicates addition of substrate. Abscissa, time in minutes; ordinate, micro liters of O_2 per egg. Graphs represent typical experiment.

give approximately the same amount (intensity) of color with the reagent (Morse, 1947; Morris, 1948).

To 3 cc. of an experimental solution, 6 cc. of "Microne" were added from a burette that permitted rapid flow of the viscous acid solution. The components of the tube were mixed rapidly and placed in a water bath (25° C. ± 0.2) 30 minutes for color development. At the end of this period, measurements were made imme-

diately using the Coleman Model IU spectrophotometer at 625 mμ. A series of standards was run with each group of experimental solutions.

Analyses were made on each of the following: (a) An acid hydrolysis of the whole egg which is recorded as the total carbohydrate (Fig. 1, curve A). These data are strikingly similar to those found by Hill (1945). (b) A saline-soluble fraction which is recorded as the carbohydrate content of the yolk (Fig. 1, curve B). This latter fraction was obtained by suspending the contents of the egg in a concentration of 1 egg/2 cc., in 0.9% saline. The suspension was then centrifuged for

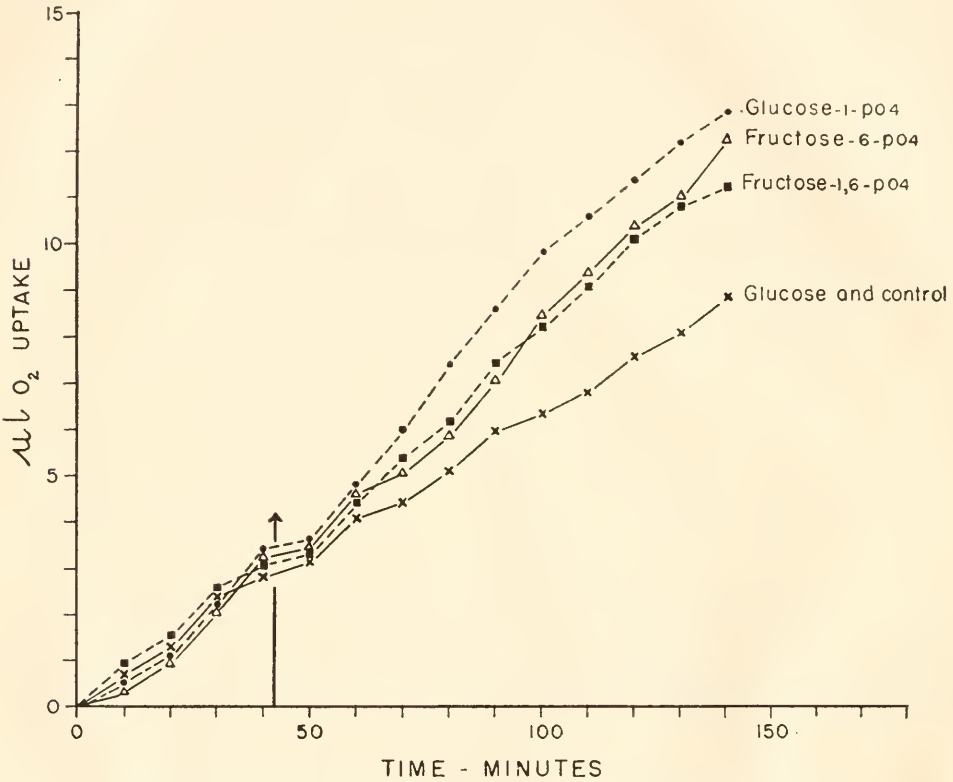

FIGURE 3. Same as Figure 2 except for homogenate of diapause embryo.

one minute at low speed to remove embryo, shell and extra-embryonic membranes, after which an aliquot was taken for analysis. (c) A trichloracetic acid (TCA)-soluble fraction which is recorded separately as the hexose content of the embryo and the yolk (Fig. 1, curves C and D, respectively). The yolk hexose was obtained by breaking the eggs in 10% TCA and then removing the intact embryos, precipitated protein and shells by centrifugation (5000 G). Lipids were removed from centrifuged samples by means of suction pipettes. An aliquot of the supernatant was diluted and used for analysis and designated as yolk hexose. The embryo hexose was determined by homogenization of saline-washed embryos in 10% TCA. Centrifugation (5000 G) was employed to remove the precipitated protein and

suction pipettes to remove the lipid layer. An aliquot was then diluted and used for analysis.

Results

The total carbohydrate content of the whole egg is graphically represented in Figure 1, A and these values are strikingly similar to those reported earlier by Hill (1945). An examination of this curve shows a marked initial increase during pre-diapause, which is probably due to the formation of carbohydrate-containing

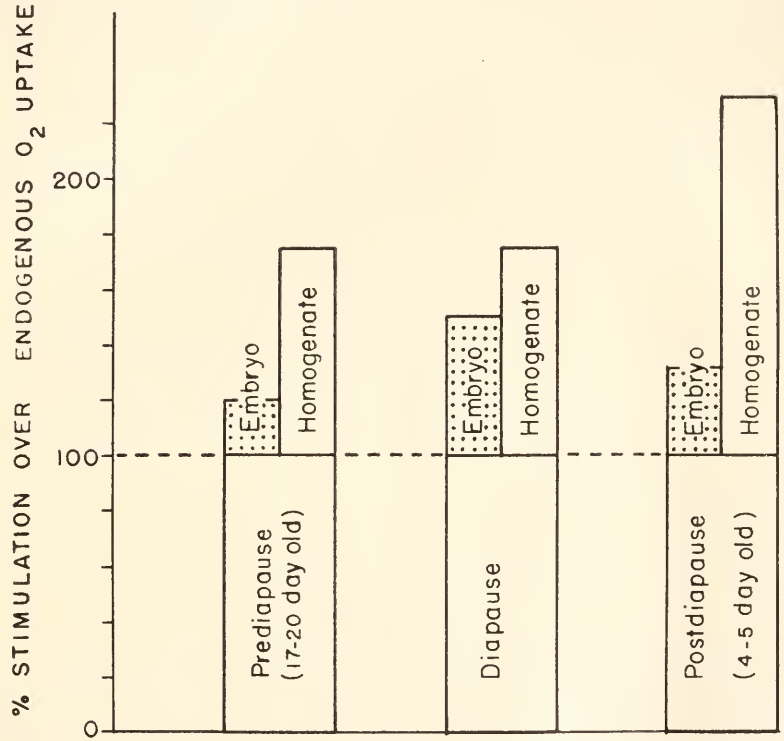

Figure 4. Shows percentage stimulation over the endogenous O_2 uptake of intact embryos and homogenates due to addition of substrates, glucose-1-PO_4, fructose-6-PO_4, in final concentration of 0.026 *M*. Stages of development indicated on abscissa; per cent stimulation on ordinate. Data taken from averages of all experiments.

cuticles. This increase is followed by a drop in total carbohydrate to a value which remains rather constant throughout the remainder of embryonic development. The general significance of the carbohydrate content of the whole egg for development has been discussed by Hill (1945) and no further reference to it will be made at this time.

The concentration of carbohydrates in the yolk is graphically presented in Figure 1, B. An examination of this curve shows that from the time of laying to about the fifth day pre-diapause, there appears to be an increase of approximately

19 μgms. of carbohydrate per egg. This rise is followed by a fall of approximately 40 μgms. carbohydrate per egg between the fifth and fifteenth days of pre-diapause development. This latter concentration is maintained throughout the remaining pre-diapause and diapause developmental periods but falls again during post-diapause (Fig. 1, B).

The concentration of the TCA-soluble carbohydrate of the yolk (Fig. 1, D) is considered to represent the hexoses. These concentrations are similar to the free reducing substances of Hill, who also suggested them to be hexoses. An analysis of curve D reveals a drop of approximately 32 μgm. carbohydrate per egg from 15-day pre-diapause to diapause. A constant level is noted during diapause and a rise of 20 μgms. carbohydrate per egg by 10-day post-diapause development. The

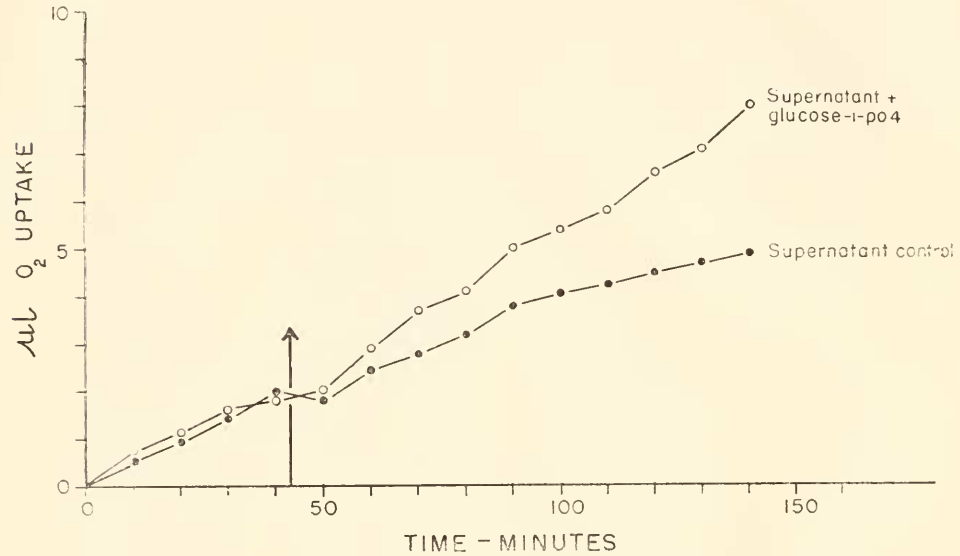

FIGURE 5. Same as Figure 2 except for cytoplasm of diapause embryo.

TCA-soluble carbohydrate (hexose) content of the embryo (Fig. 1, C) is higher in both pre-diapause and post-diapause than in diapause. This result agrees qualitatively with that presented by Hill (1945). The values recorded here, however, are approximately one-tenth of those presented by him for the free reducing substances of the embryo.

RESPIRATION OF THE ISOLATED, INTACT EMBRYO

Embryonic respiration during the mitotically active and blocked stages of development is stimulated by the addition of hexose monophosphates while glucose has no effect over a 100-minute exposure (Figs. 2 and 4). The percentage stimulation due to the hexose phosphates during diapause or blocked periods appears greater than that in pre- and post-diapause (Fig. 4). Hexose diphosphate, although not extensively investigated, seems to act similarly to hexose monophosphates.

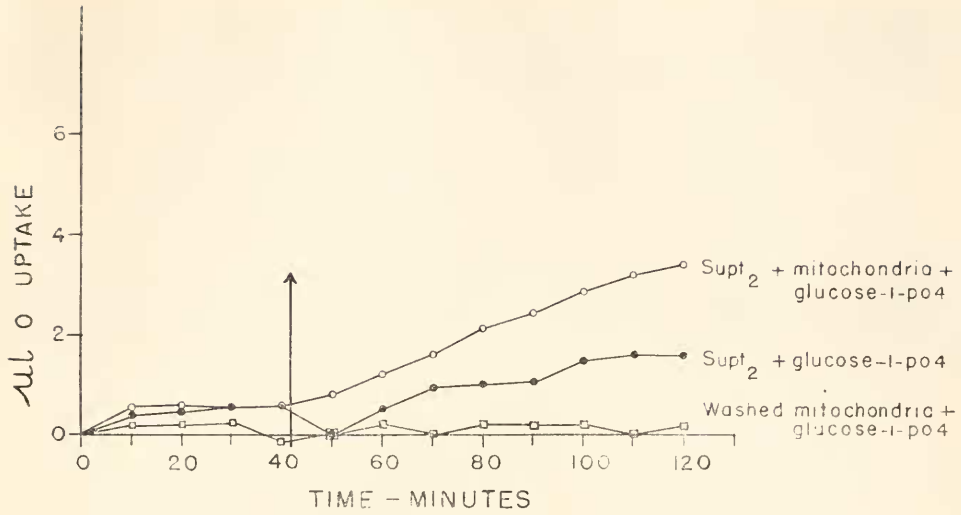

FIGURE 6. Same as Figure 2 except for fractionated cytoplasm.

HOMOGENATE RESPIRATION

The endogenous respiration of broken embryonic cells (homogenate) is markedly stimulated by hexose monophosphates in the presence of Mg^{++} (Fig. 3). Mg^{++} alone does not affect the endogenous respiration of homogenates (Fig. 3).

Hexose diphosphate shows a much lower percentage increase of stimulation of

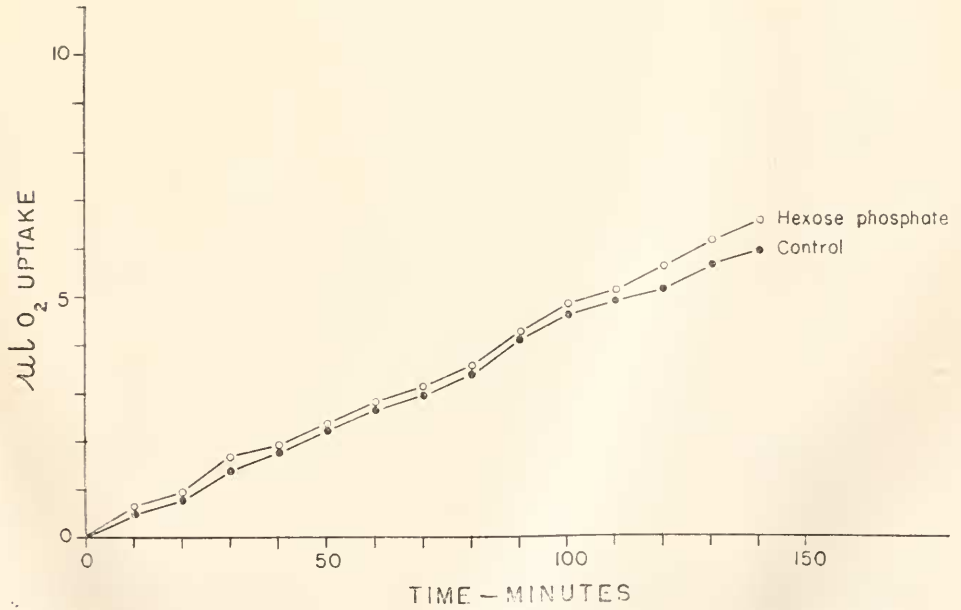

FIGURE 7. Same as Figure 2 except for isolated nuclei.

endogenous respiration than the hexose monophosphates (Fig. 3). This weak aldolase activity was unexpected and will be dealt with in a later paper.

The percentage stimulation of endogenous respiration tends to increase during post-diapause development (Fig. 4).

Fractionation and Inhibition of Cellular Respiration

As previously indicated, the supernatant or cytoplasmic fractions of the centrifuged embryo homogenates seem to contain the aerobic glycolytic enzymes (Fig. 5). When the mitochondria are removed from the supernatant the aerobic glycolytic functions are slightly reduced (Fig. 6) while the washed mitochondria show no glycolytic action whatever (Fig. 6). Mitochondria added to supernatant from

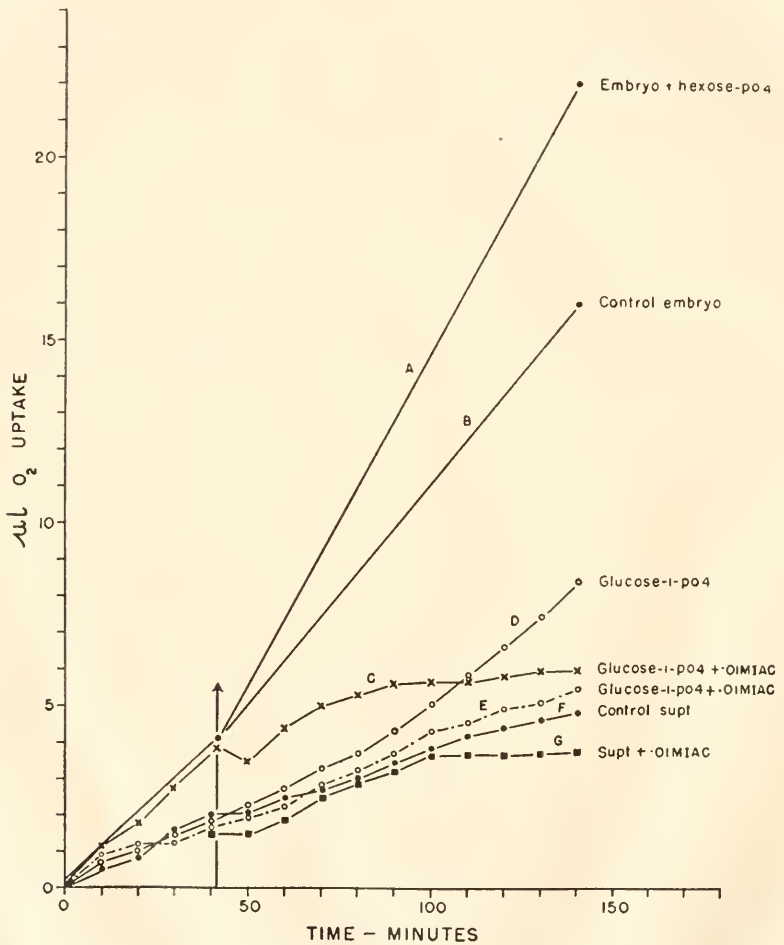

Figure 8. Shows effect of iodoacetate (IAC) on O_2 uptake of intact diapause embryo and isolated cytoplasm. A, B, C intact embryo; D, E, F, G cytoplasm. Otherwise same as Figure 2.

which they were removed tend to show an added action over the supernatant alone. Once the original fraction is broken down into its various constituents and these in turn again added together, the glycolytic action (aerobic) is greatly reduced over that of the non-fractionated supernatant. No aerobic glycolytic action occurs in the isolated nuclei (Fig. 7).

Iodo-acetate (0.01 *M*) markedly inhibits the aerobic metabolism of hexose phosphate both in the intact embryo and its cytoplasm (Fig. 8).

Discussion

Some chemical changes in carbohydrates during development of the egg and embryo of the grasshopper have been previously investigated in this laboratory, using the Hagedorn-Jensen method for reducing sugars (Hill, 1945). Hill discussed fully the changes in carbohydrates in relation to extra-embryonic membrane formation, embryonic development and energetics. He suggested that the chemical changes in the egg are consistent with Needham's theory of a definite sequence in which metabolites are used and also with the concept that terrestrial eggs during development burn fat predominantly.

It is apparent from the present results that an initial fall in carbohydrate content of the yolk between 0-day and 15-day pre-diapause is followed by no marked change in the yolk until the post-diapause period. The curve for total carbohydrates (Fig. 1, A) also reveals qualitatively similar results. Hill associates these early (pre-diapause) and late (post-diapause) changes with the carbohydrate phase of metabolism and extra-embryonic membrane formation which probably involves protein metabolism, and also suggests that the increased carbohydrates may be in part supplied by conversions of protein.

The carbohydrate lost by the yolk in post-diapause is apparently gained by the embryo since a constant carbohydrate content in the acid hydrolysis fraction exists (Fig. 1, D).

A comparison of the oxygen consumed during the stages 18–20-day pre-diapause, diapause and 4–5-day post-diapause shows that changes in carbohydrate content cannot account for the increased intensity of aerobic metabolism of the intact egg (Hill, 1945). However, the loss in hexoses (10% TCH-soluble carbohydrate) of the embryo and yolk during pre-diapause is more than sufficient to account for the increased respiratory activity of the intact embryo. Thus, carbohydrate may act as a source of energy for the marked synthesis within the embryo. This is further supported by data on the R.Q. which is slightly higher for the isolated embryo than for the intact egg.

Bodine and Boell (1934) observed that mitotically active post-diapause embryonic cells can utilize glucose, a stimulation in O_2 uptake occurring after an exposure of two hours. The results presented here for 100 minutes exposure do show that the embryo can utilize hexose phosphates to a greater extent than glucose over the same length of time (Figs. 2 and 4). Phosphorylated hexose seems to be utilized whether the cells are mitotically active or blocked, intact or broken. (The Mg^{++} ion is necessary for utilization by broken cells.) This suggests that hexokinase activity is either absent or present in very small amounts as compared with the enzymes necessary for the aerobic breakdown of these phosphorylated intermediates.

The apparent absence of hexokinase is paralleled by a lack of alkaline phosphatase activity in the embryo until approximately 8 days post-diapause. Fitzgerald (1949) suggested that alkaline phosphatase might be concerned in the transport of food material to the embryo, since consistent increase in phosphate in both pre- and post-diapause is paralleled by an increased alkaline phosphatase activity of the yolk.

The free reducing substances of Hill and the present TCH-soluble carbohydrate (Fig. 1) are a possible source of hexose, with phosphorylation perhaps occurring within the yolk itself or at the cell membrane of the embryo.

The percentage stimulation of embryonic respiration using hexose monophosphates as substrate varies markedly over the stages investigated (Fig. 4). It is greatest in diapause. However, the percentage stimulation of the homogenate does not parallel that of the embryo either qualitatively or quantitatively, being greatest in mitotically active post-diapause. This may indicate that diffusion is a limiting factor, particularly since the concentration of TCA-soluble carbohydrate is higher in 17–20-day pre-diapause and 4–5-day post-diapause than that in diapause. Further, the maximal increase in homogenate stimulation is similar for both diapause (blocked) and pre-diapause (active) while the embryonic stimulation varies, indicating perhaps a higher relative saturation of the enzyme in the intact mitotically active embryos. Moreover, the constancy of stimulation of the homogenate in pre-diapause and diapause indicates little, if any, synthesis of enzymes associated with aerobic glycolysis during this developmental period. Either a marked synthesis or increase in activity must occur at post-diapause, as shown by the great increase in stimulation of the homogenate.

Following separation of the embryo homogenate into supernatant and nuclear fractions, the enzymatic activity was found to reside primarily in the supernatant. The mitochondria (large granules) isolated from the supernatant by centrifugation were not stimulated by added hexose phosphates. The supernatant (microsomes and remaining cytoplasm) shows very weak stimulation, while a combination of the two fractions gives a higher percentage O_2 uptake but not equivalent to the original supernatant itself. Thus, the enzymes associated with aerobic glycolysis appear more intimately associated with the soluble protein fraction, as has been shown to be the case for mammalian material.

Iodoacetate, a dehydrogenase inhibitor, believed by some to be specific for aldolase activity, inhibits almost completely the stimulation of the hexose monophosphates. A recent article by Cleland and Rothschild on the oxidation of carbohydrate of the sea-urchin egg strongly suggests metabolic changes in this material similar to those for the grasshopper embryo (Cleland and Rothschild, 1952).

Summary

1. A study has been made of the carbohydrate content and changes in the intact egg and isolated embryo of the grasshopper, *Melanoplus differentialis*, during the course of embryonic development.

2. Changes in the carbohydrate content of yolk have been compared with similar changes in the developing embryo.

3. Respiration of intact isolated embryos in different carbohydrate substrates indicates a marked utilization of phosphorylated compounds.

4. The endogenous oxygen uptake of embryo homogenates is markedly stimulated by hexose monophosphates in the presence of Mg^{++}.

5. Aerobic glycolysis seems to be controlled by enzymes located only in the cytoplasm of the embryonic cells.

6. Phosphorylation of carbohydrates occurs either in the yolk or at the surface of the embryonic cells.

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